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The EGF-like receptor tyrosine kinase family is frequently overexpressed in a variety of human carcinomas, including breast cancer. To assist in characterizing the role of ErbB-4 in breast cancer, we generated three specific hammerhead ribozymes targeted to the ErbB-4 mRNA. These ribozymes, Rz6, Rz21, and Rz29, efficiently catalyzed the specific cleavage of ErbB-4 message in a cell-free system. We demonstrated that the heregulin induced mitogenic effect was abolished in ribozyme Rz29 and Rz6 transfected 32D/ErbB-4 cell. In addition, we provide the first evidence that different threshold levels of ErbB-4 expression and activation correlate with different responses to HRG stimulation. Furthermore, expression of the functional ErbB-4 ribozyme in T47D human breast carcinoma cells lead to a down regulation of endogenous ErbB-4 expression and a reduction of anchorage-independent colony formation. This preliminary result suggests that ErbB-4 plays a role in human breast cancer. We will expend our studies on investigation of the biological effect on down-regulation of ErbB-3 receptor expression by a ribozyme targeting ErbB-3 in breast cancer cells *in vitro* and *in vivo*. These studies will support the potential use of ribozymes as therapeutic agents for human breast cancers.

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Carson Tang 7/20/97

PI - Signature Date

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PART 1: P.I. Careen Tang

INTRODUCTION

Statement of rational: Interest in the ErbB family of receptors arose not only because members of the family are widely expressed in mesenchymal, neuronal and epithelial cells, but also because they are implicated in the development of human adenocarcinomas (1-3). Our previous work demonstrated that *heregulin (HRG)*, a ligand of ErbB-3 and ErbB-4, is involved in human breast cancer progression. In our proposal we plan to utilize hammerhead ribozymes which target HRG and its receptors (ErbB-3, ErbB-4) to interrupt their signaling. We will then study their roles in cell proliferation, hormone sensitivity and tumorigenicity. These results will provide us with important information relating to the biological significance of their expression in human breast cancer. These data will support the potential of using ribozymes as therapeutic agents for human breast cancer.

Background: The EGF receptor (EGFR) family is one group of tyrosine kinases that is frequently overexpressed in a variety of carcinomas (1-3). This class I subfamily of receptors is comprised of four members: HER1/EGFR (4), HER2/ErbB-2/*neu* (5-10), HER3/ErbB-3 (11, 12), and HER4/ErbB-4 (13). A number of growth factors, classified as EGF-like ligands, have been identified that can bind and stimulate the kinase activity of EGF-family receptors. EGF (14), transforming growth factor α (TGFα)(15), amphiregulin (AR)(16), heparin-binding EGF(HB-EGF)(17, 18), and betacellulin (BTC) (19) have been described as specific for EGFR. Several differentially spliced variants named heregulin (HRG), or neu differentiation factor (NDF) (20-23), acetylcholine-receptor inducing activity (ARIA) (24), glial growth factor (GGF) (25) and gp30 (26) were initially identified as candidate *neu* ligands by their ability to induce *neu* tyrosine phosphorylation (27, 28). However, recent results demonstrate that ErbB-3 and ErbB-4 are the primary receptors for heregulin (29, 30). Activation of ErbB-2 by HRG is thought to occur through transphosphorylation resulting from heterodimerization with either ErbB-3 or ErbB-4 (31, 32). Recently, betacellulin was also shown to activate the ErbB-4 receptor in a Ba/F3 system (35). Most human breast cancer cells express more than one of the EGF family receptors and different combinations of receptors can heterodimerize or homodimerize. This could activate different signaling pathways and contribute to the pathogenicity and tumorigenicity of breast cancer (28, 29, 32).

Amplification and/or overexpression of EGFR and ErbB-2 are clearly important factors in neoplastic transformation of breast epithelium (33, 34). However, ErbB-3 shows a wide range of expression in breast cancer. Overexpression of ErbB-3 is associated with the presence of lymph node metastases, but there is no correlation with patient survival at the present time (18). Elevated ErbB-4 levels have been found in certain breast cancer cell lines (31), but little is known about the expression or the clinical significance of ErbB-4 receptors in the diagnosis and prognosis of human breast cancer. It is therefore imperative that the role of ErbB-4 and its biological significance in breast cancer be defined. To achieve this goal, we employed ribozyme technology to disrupt ErbB-4 expression in human breast cancer cells.

In this report, we will discuss our research progress during the last 12 months (7/1/96-6/30/97) in investigating the biological effects of interruption of ErbB-4 pathogenic pathways by ribozymes in human breast cancer.

PART 2

BODY

Investigate the biological effect of interruption of ErbB-4 receptor pathogenic pathways in breast cancer cells.

Rationale: In our MCF-7/*HRG* transfection model and the data from others (36), we demonstrated that *HRG* expression is correlated with a more aggressive and tumorigenic phenotype. It is conceivable that the generation of ribozymes targeting the cognate receptor ErbB-4 will enable us to study the biological significance of ErbB-4 expression in breast cancer cells.

Assumptions: Introducing ribozymes which target ErbB-4 in breast cancer cells will down-regulate the endogenous ErbB-4 expression and diminish its intracellular signaling. Because of heterodimerization between the receptors of the EGF receptor family, these ribozymes will also indirectly diminish the EGFR and ErbB-2 receptor signaling pathways. Taken together, down-regulation of endogenous ErbB-4 expression may reduce the tumorigenicity in breast cancer.

Results: To assist in characterizing the role of ErbB-4 in breast cancer, we have generated three specific hammerhead ribozymes (Rz6, Rz21, and Rz29) targeted to the ErbB-4 mRNA. In previous results, we have demonstrated that these ribozymes efficiently catalyzed the specific cleavage of ErbB-4 message in a cell-free system. In the following sections (corresponding to Aim 2 in the grant), we will discuss our research findings concerning the role of ErbB-4 in breast cancer. We have carried out the first set of experiments to assess the intracellular catalytic activity of ErbB-4 ribozymes.

Rationale: Although the ribozyme sensitivity in an **extracellular** system can be correlated with the predicted secondary structure of the target RNA, the **intracellular susceptibility** of the target RNAs to ribozymes can**not** necessarily be correlated with their predicted secondary structure. There are major challenges involved in studying the efficacy of a ribozyme's ability to down-regulate **endogenous** gene expression. **1)** Subcellular compartmentalization prevents the target RNA from meeting with a ribozyme as it travels to the cytosol (37). **2)** RNA-binding proteins make the co-localization of two separate RNA strands problematic. **3)** The target site may not be accessible to the cognate ribozyme due to the splicing and refolding of the endogenous gene transcript. Most breast cancer cell lines co-express EGF family receptors. The complexity of heterodimerization and transphosphorylation between the family receptors makes it difficult to study the specificity of these ErbB-4 ribozymes in breast cancer cells. Furthermore, the goal of these ribozymes is to interrupt gene expression. If ErbB-4 is one of the critical factors involved in cell proliferation, down-regulation of this gene may be lethal to the cells. Thus, an ideal system for screening the intracellular enzymatic activity of these ribozymes requires the following criteria. **1) Expression of high levels of ErbB-4 receptor. 2) No expression of other EGF family receptors. 3) Introducion of the ErbB-4 ribozyme into these cells would not be lethal. 4) A simple bio-assay can detect the ribozyme activity.** The 32D cell system meets these requirements, and therefore was used as a model system to examine the intracellular efficacy and specificity of these

ribozymes. The following section (1) will describe this model system in detail.

(1.) An intracellular model system for studying the specificity and efficacy of these ErbB-4 ribozymes:

32D cells are a murine hematopoietic IL-3-dependent cell line that do not express detectable levels of endogenous EGF-family receptors. Studies have shown that IL-3-dependence can be abrogated by introduction of foreign growth factor receptor genes followed by stimulation with the appropriate growth factor (38). This approach exploits a unique property of this model system. If HRG can induce ErbB-4 transfected cells to bypass the IL3-dependent pathway, we can then use a simple growth assay to determine the biological function of these ribozymes intracellularly (see Figure 1).

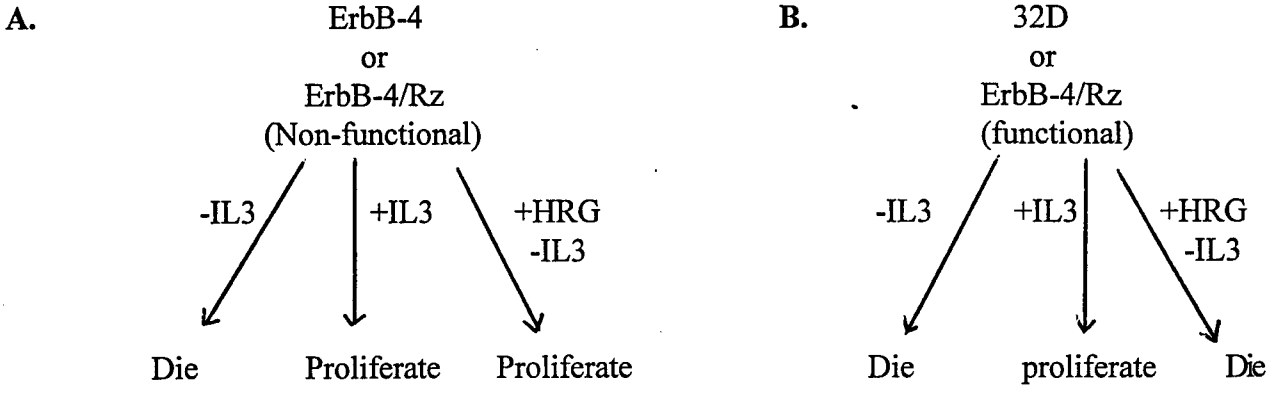


Figure 1. Schematic representations of the systems for examining ErbB-4 ribozyme efficacy and function in 32D cells.

A.) ErbB-4 expressing 32D cells (ErbB-4) will be able to bypass the IL-3-dependent pathway with cognate ligand HRG treatments. Thus, the non-functional ErbB-4 ribozyme should not change the phenotype.
B.) Illustrates that the 32D cells are absolutely IL-3-dependent and that the functional ribozyme will down-regulate ErbB-4 expression and reverse the IL-3-independent phenotype.

The beauty of this system is that it can be turned on and off. Furthermore, HRG also can rescue cells from IL-3 deprivation. **Thus, this system will allow us to study both the ribozyme efficacy and receptor function. It will also provide us a more effective and easy way to screen ribozymes.**

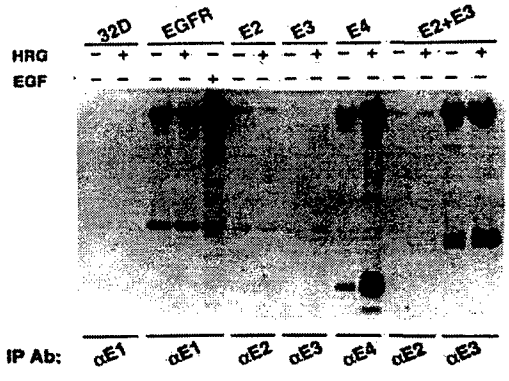
1A. Biological function of EGF family receptors in 32D cells: We have obtained the 32D cell system from J. Pierce at NIH. 32D transfected cells were established that ectopically express the EGF family receptors singly and in pairwise combinations (39). These cells were designated as 32D/E2, 32D/E3, 32D/E2+E3 and 32D/E4. (E2, E3 and E4 refer to ErbB-2, ErbB-3 and ErbB-4 receptors). The parental 32D cells do not express detectable levels of known EGF family receptors.

The 32D transfectants express high levels of corresponding receptors. The expression of the receptors in the 32D transfectants was confirmed by western blotting or immunoprecipitation followed by western blotting, and FACS analysis (data not shown). In the absence of cognate ligands, all of the 32D transfectants remained dependent on IL-3 for survival. We tested whether HRG was able to induce the IL-3-independent survival or proliferation of these 32D derivatives. We performed a growth assay, and as we expected, the untransfected parental cells were not stimulated by HRG. Cells transfected with ErbB-4 responded to HRG stimulation, bypassing the IL-3-dependent pathway (Figure 2A). We also observed that HRG can abrogate the IL-3-dependent pathway in 32D transfectants co-expressing ErbB-2 and ErbB-3 (figure 2A).

Regulation of tyrosine phosphorylation of each receptor by HRG was evaluated by immunoprecipitating the corresponding receptors and immunoblotting with anti-phosphotyrosine. Figure 2B demonstrates that no autophosphorylation was observed in the parental cells (32D) in the presence of HRG. In ErbB-4 expressing cells, the receptors were constitutively phosphorylated; however, phosphorylation could further be induced by exposure to its cognate ligand. In 32D/E2+E3 cells, we observed a high basal level of phosphorylated ErbB-3, and were able to further induce phosphorylation by HRG. Thus, this is **an ideal system to study the specificity and efficacy of the ribozymes targeting the ErbB- family receptors. This system can be turned on and off by IL-3 or the appropriate ligands.** Therefore, a simple growth assay will able to define the intracellular enzymatic activity of ribozymes.

Figure 2B. Regulation of receptor tyrosine phosphorylation by HRG in 32D/E4 and 32D/E2+E3 cells.

500ug of lysates from untreated '-' or HRG (100ng/ml for 5 minutes) treated '+' 32D transfectants (32D/wt, 32D/E2, 32D/E3, 32D/E4, 32D/E2+E3) were immunoprecipitated with anti-receptor antibodies (αE2, αE3, αE4). The 32D/EGFR cells (E1) were treated with 100ng/ml of EGF for 5 minutes and immunoprecipitated with anti-EGFR antibody (αE1). The precipitates were then subjected to western blotting with an anti-phosphotyrosine antibody (UBI). Comparative amounts of receptor proteins were used in the phosphorylation assay and confirmed by western blot with corresponding antibody.



Conclusion: The 32D cell system is an ideal system to study the specificity and efficacy of the ribozymes targeting the ErbB- family receptors. Section 2 will demonstrate the ErbB-4 ribozyme catalytic activity in the 32D cell system.

(2.) Demonstration of ErbB-4 ribozyme catalytic activity in 32D cells:`

2A. ErbB-4 ribozymes abolish HRG induced IL-3-independence: We constructed all three of these ErbB-4 ribozymes in a mammalian expression vector under a CMV promoter control. We then transfected the ErbB-4 Rz into 32D/E4 cells. We hypothesized that the **functional ribozymes would down-regulate ErbB-4 expression and thereby reduce or abolish the HRG induced, IL-3-independent survival or proliferation** (see Figure 3). We performed a growth assay in the presence and absence of HRG using these ErbB-4 Rz transfected cells. We observed that one of the ErbB-4 ribozymes (Rz29) is capable of reversing the HRG induced IL3-independent phenotype compared with the control cells (32D/E4) and the vector transfected cells. Rz6 can partially inhibit the HRG effect. Rz21 has no effect. **This result is unlikely due to low expression of Rz21, since all these ribozymes used the same expression vector. It is possible that the target site is not accessable to Rz21.** Table 1 summarizes the ribozyme effects in these ErbB-4 cells. We then evaluated the specificityof these ErbB-4 ribozymes to rule out cross-reactivity of the ErbB-4 ribozyme with other family members. We transfected all three ErbB-4 ribozymes into 32D/E2+E3 derivative cells. We did not observe any effect on the HRG induced IL3-independent phenotype.

These data suggest that Rz6 and Rz29 are functional ribozymes, and that the effects of these ErbB-4 ribozymes are highly specific for the ErbB-4 receptor. Rz29 exhibits a higher level of biological activity compared to Rz6. Rz21 is apparently a non-functional ribozyme in 32D cells.

2B. ErbB-4 ribozyme abolishes HRG stimulation of mitogenesis: We also assessed the mitogenic response of these ErbB-4 Rz transfected cells to confirm the above result. We performed a mitogenic assay to observe DNA synthesis in these ErbB-4 Rz transfected cells. As shown in figure 3, in the absence of IL-3, all the 32D derivative cells exhibit very low levels of thymidine incorporation. However, in the presence of IL-3, all the 32D derivative cells exhibit high levels of ^{3}[H] thymidine incorporation as expected. In the control cells (32D/E4), HRG was able to stimulate high levels of ^{3}[H] thymidine incorporation in the absence of IL-3, whereas the ^{3}[H] thymidine incorporation was almost completely abolished in the Rz29 transfected cells. The ^{3}[H] thymidine incorporation was significantly reduced in the Rz6 transfected cells. No significant changes in the Rz21 transfected cells was seen (data not shown). **These results correlate with the growth assay.**

2C. ErbB-4-Rz mediated down-regulation of ErbB-4 expression levels in 32D/ErbB-4 cells: To evaluate the intracellular enzymatic cleavage effects of ErbB-4 ribozymes on ErbB-4 expression, the ribozyme transfectants were examined on their target mRNA level. A northern blot analysis was performed. We observed that Rz6 and Rz29 were able to down-regulate the ErbB-4 mRNA level significantly in these ribozyme transfected cells. Rz21 did not have any significant effect (data not shown). Thus, the abolishment of the HRG-induced IL-3 independent biological effect correlates with reduction of ErbB-4 mRNA levels in these cells.

To further characterize the ribozyme effect, we quantitatively examined the ErbB-4 ribozyme mediated down-regulation of ErbB-4 receptor expression in these ErbB-4Rz transfected cells by FACS analysis. The results, depicted in figure 4, show that the ErbB-4 transfected cells express high levels of ErbB-4 receptor. **Rz29 down-regulates ErbB-4 expression by 65%, Rz6 down-regulates**

ErbB-4 expression by 45% and no significant effect was detected in Rz21 transfected cells. These data suggest that the constructed ErbB-4 Rz29 and Rz6 are biologically functional ribozymes.

2D. Reduction of autophosphorylation by ErbB-4 ribozymes: To determine whether the abolishment of the HRG-induced IL-3 independent biological effect correlates with a decrease in receptor tyrosine phosphorylation, the autophosphorylation of the receptors in these cells was examined using a kinase assay. Figure 5 demonstrates that Rz29 dramatically reduced the level of ErbB-4 intrinsic tyrosine kinase activity. Because ErbB-4 expression was down-regulated only 65% by Rz29, the cells still express ErbB-4 receptors. HRG is therefore still able to induce the phosphorylation of the remaining ErbB-4 receptor. However, the level of phosphorylation was significantly lower than the 32D/E4 cells or the vector transfected cells (32D/E4/V). **Reduction of phosphorylation correlates with the expression level of ErbB-4**. Furthermore, these data also imply that while Rz29 is specifically cleaving its target mRNA, it does not affect the function of those receptors that are expressed. **Results generated in the intracellular expriments described above prove that the decrease in ErbB-4 protein production, activation and mRNA expression correlate with the ErbB-4 ribozyme catalytic activity.**

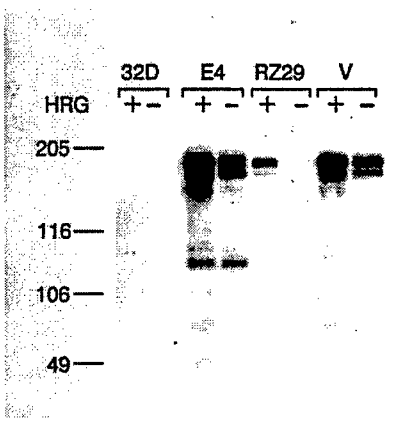


Figure 5: Reduction of autophosphorylation of ErbB-4 receptor by Rz29 ribozyme. Cells were treated with or without HRG (100ng/ml) for 5 minutes prior to lysis. 400ug of lysates were immunoprecipitated with a specific *anti-ErbB-4* antibody. No detectable level of tyrosine phosphorylation was observed in 32D parental cells (32D) which do not express any ErbB-family receptors. The level of ErbB-4 intrinsic tyrosine kinase activity in Rz29 transfected 32D/E4 cells (Rz29) was markedly reduced compared to control transfectants, ErbB-4 transfected 32D cells (32D/E4), and vector transfected 32D/E4 cells (32D/E4/Vector).

Conclusion: Our preliminary data show that these ErbB-4 ribozymes effectively catalyze precise cleavage of their target RNA sequence in an extracellular system. The constructed ErbB-4 Rz29 and Rz6 are biologically functional ribozymes and are highly specific for the targeted ErbB-4 mRNA in 32D cells. In the following section (3), we will demonstrate the effect of down-regulation of the ErbB-4 receptor by the ErbB-4 ribozyme in human breast cancer cells.

(3.) The effect of down-regulation of ErbB-4 receptor in human breast cancer cells:

To investigate the biological and biochemical functions of ErbB-4 in human breast cancer, we expressed the ErbB-4 ribozymes in several ErbB-4- positive human breast cancer cell lines. One of the cell lines is T47D, derived from a breast carcinoma. The T47D cells express moderate levels of all the currently known ErbB- receptors. We transfected the ErbB-4 ribozymes (Rz6, Rz21, Rz29) into T47D cells and selected the transfectants using G418. We observed a reduction of G418-

resistant colony formation when the Rz6 and Rz29 constructs were transfected, suggesting that down-regulation of ErbB-4 receptor in T47D cells may be lethal. We partially characterized the pooled population of the Rz6- and Rz29- transfected cells. We detected 45-50% down-regulation of ErbB-4 receptor in these cells using FACS analysis (Figure 6), whereas no effect on the level of EGFR, ErbB-2, or ErbB-3 receptors in these cells was observed. In addition, we observed that anchorage-independent colony formation was significantly reduced (45%) in the ribozyme transfected cells (Figure 7).

Conclusion: These preliminary data suggest that Rz6 and Rz29 are able to down-regulate the endogenous ErbB-4 receptor and thereby inhibit colony formation in T47D cells. The ErbB-4 receptor may therefore play a role in T47D cell proliferation.

CONCLUSION

Using the 32D cell system to study the intracellular enzymatic activity of ErbB-4 ribozymes, we clearly demonstrate that the ribozymes are specific and can effectively down-regulate the ErbB-4 receptor. In this system, one ErbB-4 ribozyme (Rz29) significantly reduced the ErbB-4 mRNA level and down-regulated ErbB-4 receptor expression (figure 4), thereby reversing the HRG-induced IL3-independent phenotype of 32D/E4 cells (table 1). Rz6 partially down-regulated the expression of the ErbB-4 receptor, and somewhat blocked the IL3-independent phenotype. In contrast, Rz21 failed to down-regulate the ErbB-4 expression or to inhibit the mitogenic response to HRG treatment in 32D/ErbB-4 cells. It is clear from these data that not all of the sites tested are equally amenable to intracellular ribozyme-mediated cleavage.

Using the ErbB-4 ribozymes in the 32D cell system, we provide the **first evidence** that different threshold levels of ErbB-4 expression and activation correlate with different responses to HRG stimulation. High levels of ErbB-4 expression, phosphorylation and homodimerization are necessary for HRG stimulated IL3-independent cell proliferation in the 32D/E4 cells. Low levels of ErbB-4 expression allow HRG-induced phosphorylation, but are insufficient to couple the receptor activation to cellular signaling, particularly in the case of Rz29-transfected 32D/E4 cells. These results also suggest that homodimers of ErbB-4 can transmit biological signals. This is consistent with a previous report that ErbB-4 homodimers constitute a functional HRG receptor (24).

To evaluate the effects of down-regulation of ErbB-4 in an ErbB-4-positive human breast cancer line, Rz29 was transfected into T47D cells. Down-regulation of ErbB-4 receptor in T47D cells resulted in a reduction in transfection efficiency and colony formation in an anchorage-independent assay compared to vector- or Rz21-transfected cells. The low efficiency of Rz6 and Rz29-expressing drug-selected clones is unlikely due to non-specific effects, since all the ribozymes were cloned into the same vector. Furthermore, Rz6 and Rz29 down-regulated ErbB-4 but not other ErbB-receptor family members. Reduction of colony formation suggests that ErbB-4 expression and mitogenic signaling may be essential for T47D cell survival. These preliminary findings suggest that down-regulation of ErbB-4 expression, as shown by FACS, diminished the ErbB-4 mediated intracellular signaling. Because of heterodimerization between the family receptors, down-regulation of ErbB-4 receptor may have also indirectly interrupted receptor signaling pathways initiated by other family members. This could result in diminished tumorigenicity in T47D cells. These results also show that our ribozyme is active in a human carcinoma cell line.

Future Directions:

We will continue to study the role of ErbB-4 in human breast cancer *in vitro* by testing the ErbB-4 ribozyme in different breast cancer cell lines and will expand these studies using an inducible promoter system. We will also elucidate the effects of these ribozymes on tumorigenicity *in vivo*.

We will extend the ErbB-4 studies to ErbB-3 to explore the pathogenic role of ErbB-3 in human breast cancer cells, using an approach similar to that in these ErbB-4 studies. We will generate the ErbB-3 ribozymes and study the effects of down-modulation of endogenous ErbB-3 levels in breast cancer cells *in vitro* and *in vivo*.

Materials and Methods:

Cell lines and cell culture: The 32D murine hematopoietic cell line (40) and its derivatives were grown in RPMI (Cellgro) supplemented with 12% fetal calf serum (Biofluids) and interleukin-3 (IL-3) supplied as 6% conditioned medium from the WEHI-3B murine mylomonocytic leukemia cell line.

Transfection by electroporation: 1 x 10^7 32D derivative cells were used for each transfection. 10ug of plasmid DNA was added to cells suspended in 300ul of PBS. Cells were electroporated at 250 volts, using a BioRad electroporation system, plated onto 100mM dishes, and incubated for 24 hr. The cells were then selected in growth medium containing 750 ug/ml geneticin (G418-sulfate, Gibco).

Northern blot analysis: Total RNA isolation using RNazol B(Tel-Test, Inc. Texas). 20ug of total RNA from each cell line was used to hybridize with an ErbB-4 cDNA probe and autoradiographed for 48 hr.

Autophosphorylation of ErbB-family receptors: A total of 2 x 10^8 32D derivative cells were washed in phosphate-buffered saline (PBS) and resuspended in 50 ml of RPMI supplemented with IL-3, and incubated for 4 hr. at 37 °C. Following incubation, cells were washed in PBS, and resuspended in 1 ml of PBS with $Na_3(VO)_4$. Remaining steps were performed on ice. Recombinant heregulin β3 isoform (EGF-like domain) was added at a final concentration of 150ng/ml. Following a 10 min incubation, cells were lysed in "Hepes-Lysis buffer" and the cell debris was pelleted by centrifugation (46).

The lysates were then immunoprecipitated with either anti-EGFR (Ab-1, Oncogene Science, Uniondale, NY), anti-*ErbB-2* (Ab-3, Oncogene Science, Uniondale, NY), anti-*ErbB-3* (C17, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-*ErbB-4* (C18, Santa Cruz Biotechnology, Santa Cruz, CA) in combination with protein-A agarose (Pharmacia, Piscataway, NJ) overnight at 4°C with gentle agitation. Detail see elsewhere (43).

Fluorescence-activated cell sorter (FACStar) analysis: 1 X 10^6 cells were harvested and then stained for one hour with an anti-ErbB-4 monoclonal antibody (Ab-1, NeoMarker), then a secondary FITC-anti-mouse antibody was used and the ErbB-4 level in each cell was quantitatively measured by flow-cytometry.

Anchorage-independent growth assay: A bottom layer of 0.1 ml IMEM containing 0.6% agar and 10% FCS was prepared in 35mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per dish) were added in a 0.8 ml top layer 0.4% Bacto Agar, and 5% FCS. All samples were prepared in triplicate. Cells were incubated for approximately 12 days at 37°C. Colonies larger than 60um were counted in a cell colony counter (Ommias 3600, Imaging Products Int., Inc. Charley, VA).

Mitogenic assay: 32D transfected cells were plated at a density of 1X10^4 cells with or

without IL-3 supplement, or supplemented with 100ng/ml of HRG in the absence of IL-3. Two days post plating, the cells were labeled with ^{3}[H] thymidine for two hours. ^{3}[H] thymidine incorporation was then analyzed by β-scintillation counter.

In vitro Kinase Assay: 32D/E4, 32D/E4+V and 32D/E4+Rz29 cells were serum starved for 2 hours prior to treatment with or without 100ng/ml of HRG. Cells were then lysed in lysis buffer. 400ug of total protein from each cell line was used to immunoprecipitate with anti-ErbB-4 antibody (C18, Santa Cruz Biotechnology, Santa Cruz, CA) in combination with protein-A sepharose (Pharmacia, Piscataway, NJ). Reactions were carried as described previously (42). 50μl of a solution containing 10mM Tris-HCL, pH 7.5, 10mM $MgCl_2$, 10mM $MnCl_2$, 10 μCi[γ-^{32}P]ATP and 1μg aprotinin was added to the washed beads for 25 min at room temperature. Reactions were terminated by spinning down the sepharose beads in a micro centrifuge, discarding the supernatant and resuspending the beads in 50μl SDS gel loading buffer. Eluted proteins were analyzed by SDS-PAGE and autoradiography.

Legends:

Table 1. Effect of ErbB-4 ribozymes on the density of 32D/E4 cells under the conditions of IL-3 starvation and HRG stimulation.

Figure 2A. Heregulin can induce IL-3-independent growth in 32D/E4 and 32D/E2+E3 cells. Growth assay: 32D cells were plated at a density of $1X10^4$ cells/ml in IL-3 free medium, medium supplemented with IL-3, or in medium lacking IL-3 but supplemented with 100ng/ml of human recombinant HRG. Viable cells were counted on day 3 after seeding.

Figure 3. ErbB-4 ribozyme abolishes HRG-induced mitogenesis. 32D transfected cells were plated at a density of $1X10^4$ cells with or without IL-3 supplement, or supplemented with 100ng/ml of HRG in the absence of IL-3. Two days after plating, the cells were labeled with ^{3}[H] thymidine for two hours. ^{3}[H] thymidine incorporation was then analyzed by scintillation counter. The parental 32D cells are labeled as 'wt'. 32D/E4 transfected cells are denoted as E4. E4+V represents the empty vector transfected 32D/E4 cells. Ribozyme transfected cells are marked as Rz6, Rz2, and Rz29. Rz29 abolished the HRG induced IL-3-independent mitogenesis. Rz6 reduced HRG induced mitogenesis significantly. Rz21 has no effect.

Figure 4: Rz29 down-regulation of ErbB-4 expression in 32D/ErbB-4 cells. The levels of ErbB-4 in 32D/E4 and Rz29 transfected 32D/E4 cells were quantitatively measured by flow-cytometry. $1X10^6$ cells were harvested and stained with an anti-ErbB-4 monoclonal antibody in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACScan. A) Expression of ErbB-4 in vector transfected cells (E4/V). Right-hand curves, specific staining; left-hand curves, nonspecific staining (primary antibody omitted); ordinates, relative cell number; abscissas, log fluorescence. B) Rz29 down-regulates ErbB-4 expression by 65%. The dotted-line curve represents the ErbB-4 expression in ErbB-4/V cells. The solid line curve represents the ErbB-4 expression in Rz29 transfected cells. C) Rz 21 has no effect on ErbB-4 expression. The dotted-line curve represents the ErbB-4 expression in ErbB-4/V cells. The solid line curve represents the ErbB-4 expression in Rz21 transfected cells. D) Rz6 down-regulates ErbB-4 expression by 45%. The dotted-line curve represents the ErbB-4 expression in ErbB-4/V cells. The solid line curve represents the ErbB-4 expression in Rz6 transfected cells.

Figure 6. ErbB-4 ribozyme down-regulation of endogenous ErbB-4 expression in T47D human breast cancer cells. The levels of ErbB-4 in T47D/wt and T47D/Rz pool clones were quantitatively measured by flow-cytometry. $1X10^6$ cells were harvested and stained with an anti-ErbB-4 monoclonal antibody in combination with fluorescence-labeled anti-mouse IgG antibody and analyzed by FACScan. Left-hand curve (thin line curve) represents nonspecific staining (primary antibody omitted). Right-hand curve (bold line curve) represents the ErbB-4 expression in T47D wild-type cells. The dotted-line curve (middle curve) represents the ErbB-4 expression in Rz6 transfected cells. The ordinates, relative cell number; abscissas, log fluorescence.

Figure 7. Expression of ErbB-4 ribozyme in T47D cells (T47D/Rz6 pool clone) inhibits colony formation by more than 50%. Anchorage-independent growth assay: A bottom layer of 0.1 ml IMEM containing 0.6% agar and 10%FCS was prepared in 35mm tissue culture dishes. After the bottom layer solidified, cells (10,000 per dish) were added on a 0.8 ml top layer containing 0.4% Bacto Agar, and 5% FCS. All samples were prepared in triplicate. The cells were incubated for approximately 12 days at 37°C. Colonies larger than 60um were counted in a cell colony counter.

Table 1. Effect of ErbB-4 ribozymes on the density of 32D/E4 cells in response to IL-3 starvation and HRG stimulations

Cell Line	Number of Viable Cells (x 1000 cells/ml)		
	-IL3	+IL3	+HRG/-IL3
E4	**1.3**	**1996**	**1490**
E4/Vector	**1**	**1894**	**1369**
E4/Rz6	**1.1**	**1717**	**367**
E4/Rz 21	**1**	**1845**	**1300**
E4/Rz 29	**1.2**	**1823**	**56**
E4/ErbB-2 ribozyme	**1.2**	**1798**	**1279**
E2+E3/Rz6	**1.1**	**1869**	**1307**
E2+E3/Rz21	**1**	**1946**	**1377**
E2+E3/Rz29	**1.2**	**1854**	**1298**

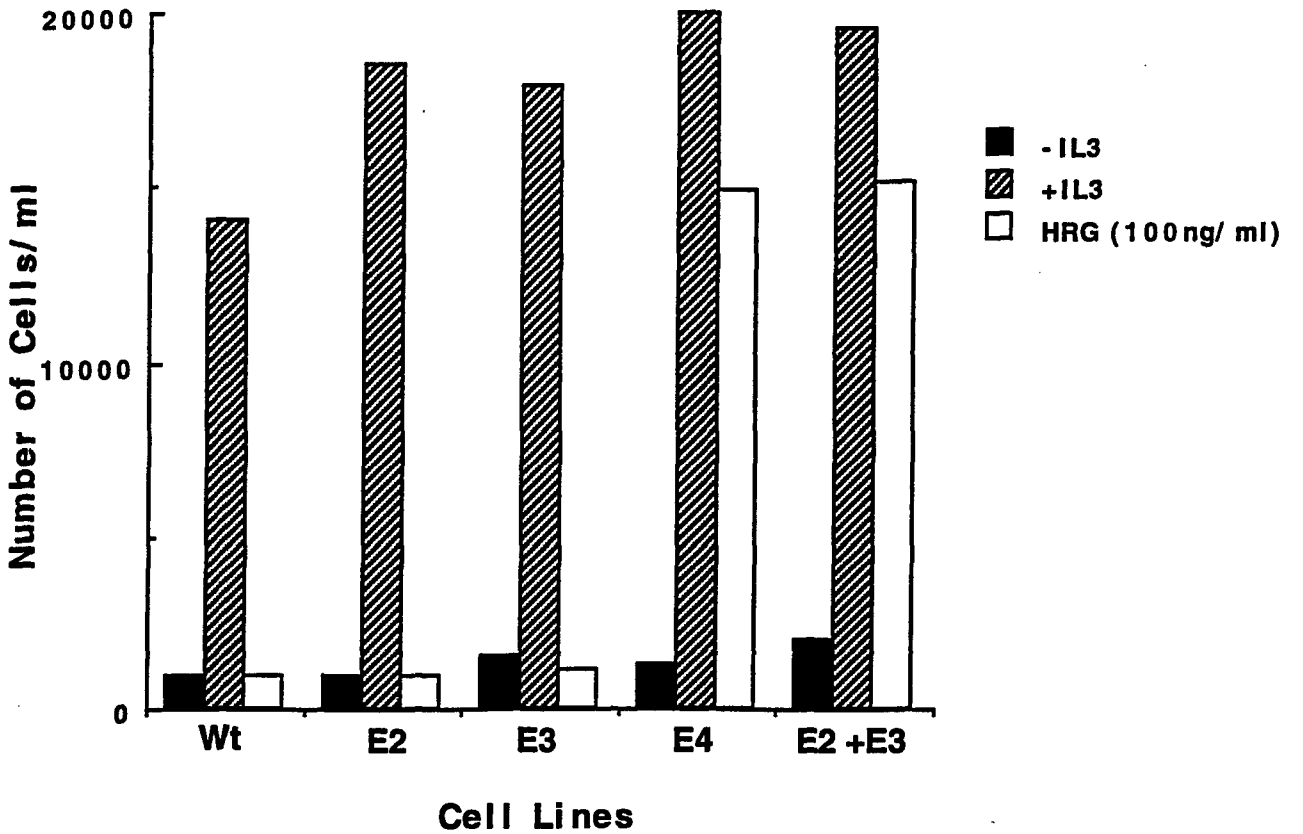

20000
10000
0
Number of Cells/ ml
Wt
E2
E3
E4
E2 +E3
Cell Lines
-IL3
+IL3
HRG (100ng/ ml)

Figure 2A

Figure 3

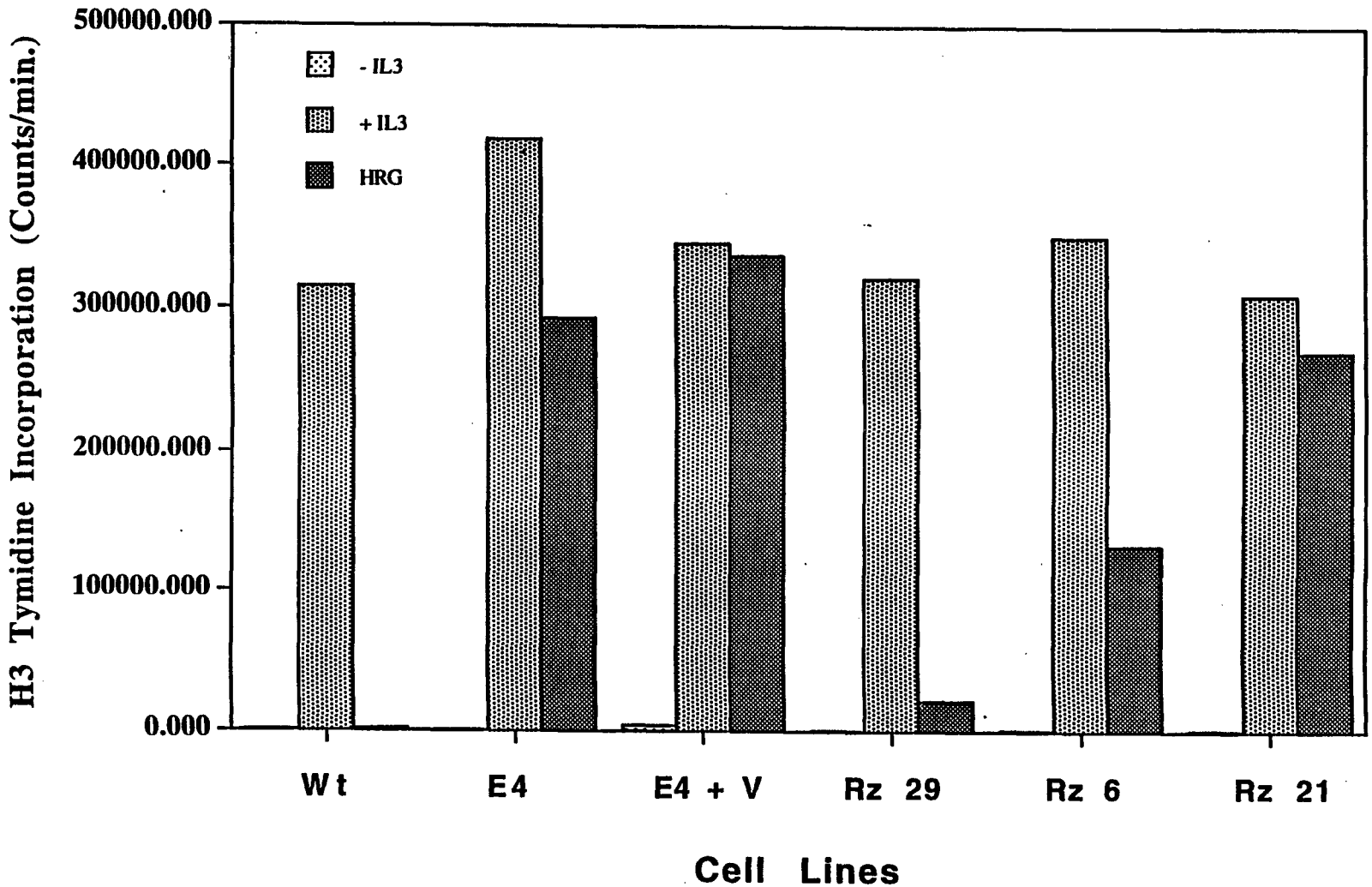
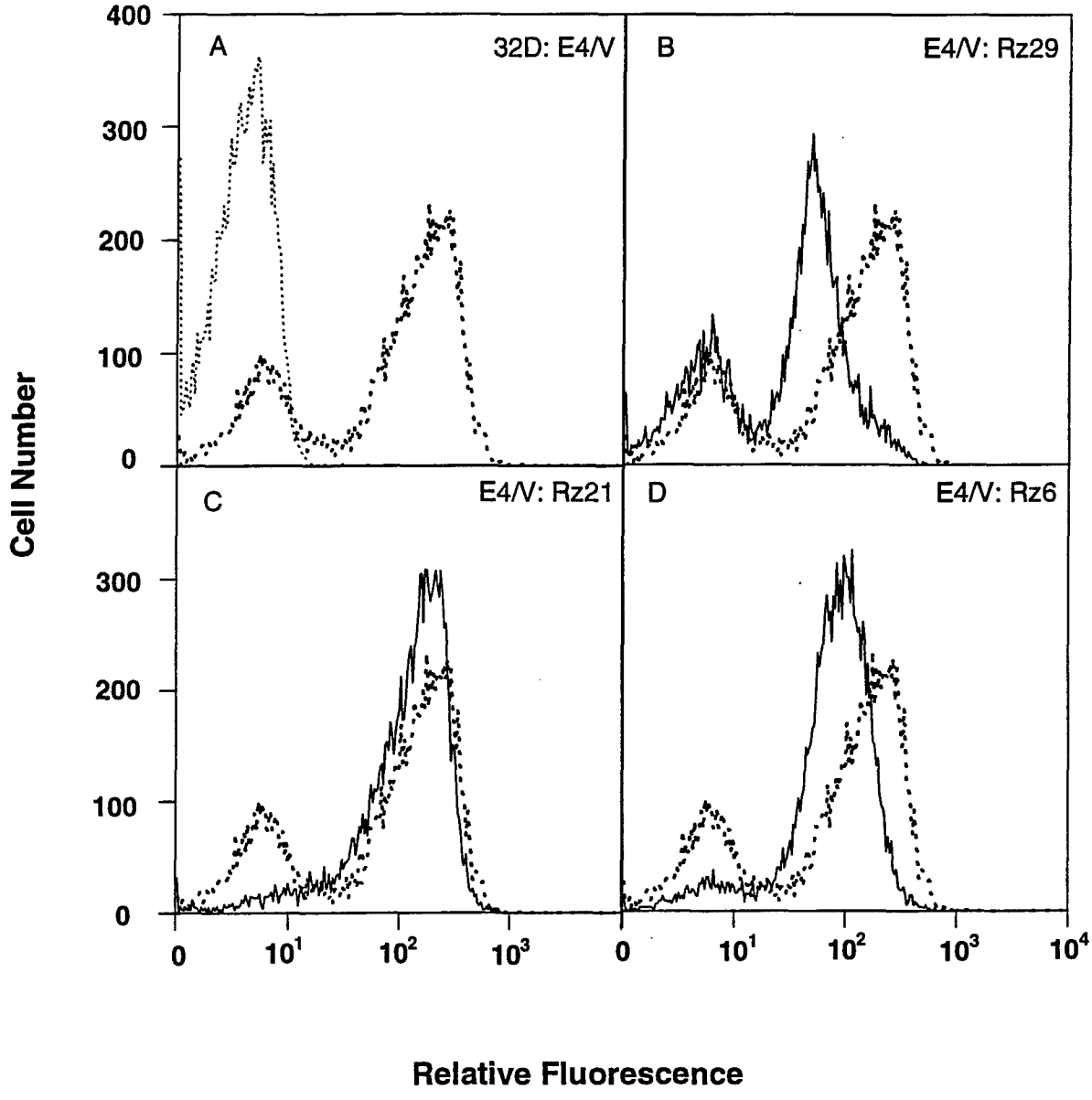

500000.000
400000.000
300000.000
200000.000
100000.000
0.000
H3 Tymidine Incorporation (Counts/min.)
- IL3
+ IL3
HRG
Wt
E4
E4 + V
Rz 29
Rz 6
Rz 21
Cell Lines

Figure 4


A
32D: E4/V
B
E4/V: Rz29
C
E4/V: Rz21
D
E4/V: Rz6
Cell Number
400
300
200
100
0
0
10^1
10^2
10^3
10^4
Relative Fluorescence

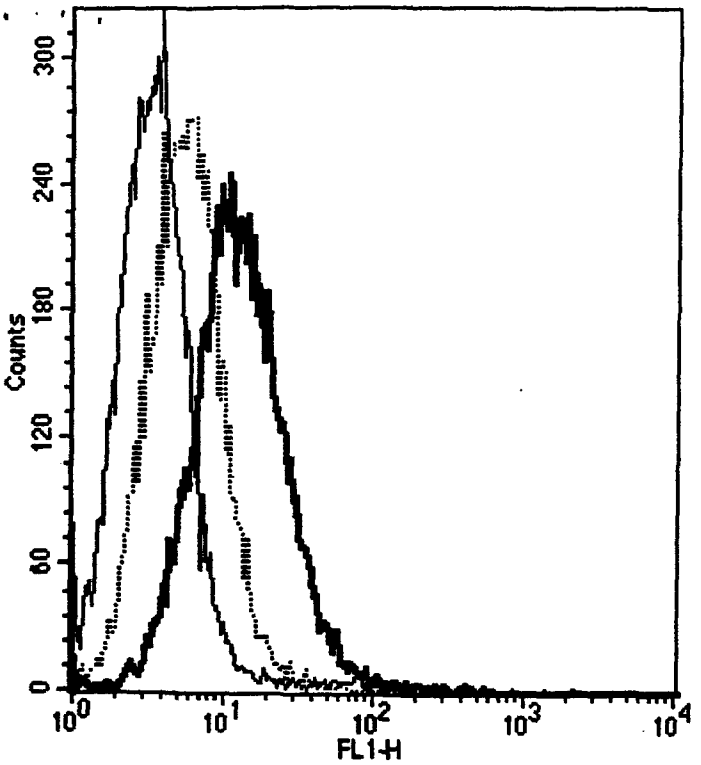


Figure 6

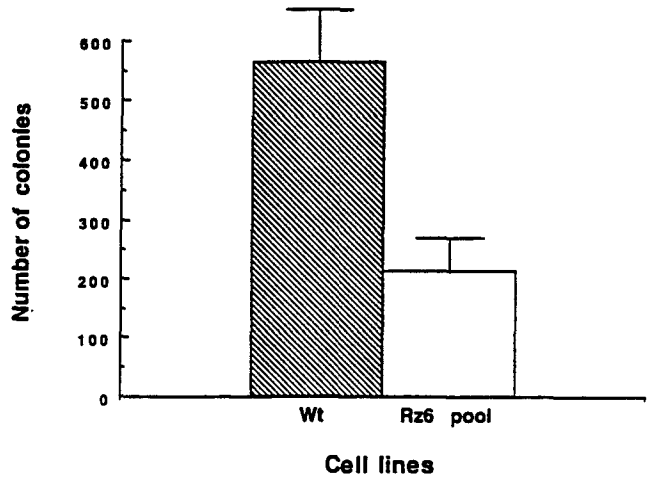

Number of colonies
600
500
400
300
200
100
0
Wt
Rz6 pool
Cell lines

Figure 7

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
Tang, Careen K.	Research Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training).*

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University of Maryland, College Park, MD	B.S.	1983	Chemistry
The Johns Hopkins University, Baltimore, MD	Ph.D.	1989	Molecular Biology/ Chemistry

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list, in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List, in chronological order, the titles, all authors, and complete references to all publications during the past three years and to representative earlier publications pertinent to this application. If the list of publications in the last three years exceeds two pages, select the most pertinent publications. **DO NOT EXCEED TWO PAGES.**

RESEARCH EXPERIENCE:

8/95 - data **Research Assistant professor** Department of Biochemistry. (Dr. Marc Lippman)
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1996 **Gold Standard Proposal** for 1995 DoD Breast Cancer Research Program

1995 **Outstanding Poster** for 1995 AACR annual meeting

1994 **Travel Award** by Bristol-Myers Oncology Division for 1994 AACR presenter of outstanding abstract.

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Publications:

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